ORIGINAL ARTICLE FOOD ALLERGY AND ANAPHYLAXIS

Children monosensitized to pine nuts have similar patterns of sensitization

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Abstract

Background: Several cases of pine nut allergies and anaphylaxis have been reported in the literature, but only few pine nut allergens have been characterized. The aim of this research is to identify through immunoelectrophoretic techniques the major pine nut allergens in a group of children monosensitized to pine nuts.
Methods: We studied five children with pine nut allergies and no other sensitization to food except to pine nuts, confirmed by *in vivo* (prick test, prick-to-prick) and *in vitro* tests (specific IgE determinations [CAP-FEIA]). The protein profile of pine nuts was analyzed by Sodium Dodecyl sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Immunoblotting was performed after incubation of membranes with the sera from the children included in the present study.
Results: Immunoblotting (SDS-PAGE) demonstrated five similar bands between 6 and 47 kDa in all the subjects studied.
Conclusion: These bands should be considered the potential allergens for pine nut allergic children.

Food allergy is a common disease in childhood with increasing prevalence (1, 2) and relative high risk of anaphylactic reactions, which are difficult to manage from both a therapeutical (3–5) and a psychological point of view (6, 7). In particular, nut allergy (8) such as peanut, tree nut, hazel nut, cashew nut, and pistachio nut, may provoke severe allergic reactions (9).

Pine nuts are the seeds of *Pinus pinea*, which is a typical conifer in southern Europe, especially in Italy, southern France and Spain. Nowadays, there is a renewed interest in pine nuts as a food, and they are consumed raw or toasted, and are included as ingredients in salads, pastries and sauces such as the typical Italian sauce 'Pesto alla Genovese.' Although several cases of pine nut allergies and anaphylaxis have been reported in the literature (10–16), only few pine nut allergens have been characterized (17–21). Moreover, some studies have described cross-reactivity between pine seeds and other nuts (22, 23) or pine pollen (24). Taking into account the previous information, the aim of this research is to identify through immunoelectrophoretic techniques the major pine nut allergens in a group of children monosensitized to pine nuts. The serological characterization of these children's sera will provide information about the specificity and sensitivity of the *in vitro* diagnostic tests and clinical symptoms, and the risk of IgE cross-reactions with other tree nuts.

Material and methods

Patients

The sera of five children (three males and two females) with history of immediate (<1 h) allergic reactions following the ingestion of pine nuts and positive skin prick tests and/or serum specific IgE to pine nuts were collected at the Department of Pediatrics, Meyer Hospital (Florence, Italy); participant age ranged from 5 to 13 yrs (mean [s.d.] age, 11.90 [3.53]). Anaphylaxis was diagnosed according to the clinical criteria proposed by Sampson et al. (25). Informed consent was obtained from the parents of all children participating in this study. This study has been performed according to the Declaration of Helsinki, and it has been approved by A. Meyer's ethics committee.

Skin tests

Skin prick tests were performed with common allergens (mites, pollens, molds, and epithelia) and food (egg, milk, wheat, corn,

tree nut, hazelnut, pine nut, pistachio nut, peanut, cod, sole, snail, shrimp, peach, apple, kiwi, carrot, tomato) of commercial origin (ALK Abelló, Madrid, Spain). Saline was used as a negative control and 10 mg/ml histamine phosphate as a positive control. Results were read after 15 min. Reactions were considered to be positive if the largest diameter of the wheal was 3 mm over the negative control. Skin tests with fresh nuts (peanut, pine nut, tree nut, almond, cashew nut, pistachio) were also performed by prick-to-prick method according to Ortolani et al. (26) in the patients and in 10 control subjects.

Specific IgE determination

Specific IgE against a panel of most common food allergens (milk, egg, cod, wheat, peanut) and nuts (tree nut, hazelnut, almond, pine nut, pistachio nut) was determined by CAP (CAP System, Pharmacia, Uppsala, Sweden).

Preparation of pine nut samples

Pine nuts (Conad, Bologna, Italy) were purchased from a local supermarket and were ground to minute particles using a coffee mill. The protein content of the resultant flour was determined according to the method described by Lowry et al. (27). Pine nut flour was then defatted by adding diethyl ether for 2 h at 4°C. After centrifugation (5000 revolution per minute, 20 min), the supernatant was discarded and the precipitate was extracted twice more. Afterward, the defatted flour was suspended in a sample buffer (containing 0.125 M TRIS–HCl pH 6.8, 3.75% glycerol, 1% SDS, 5% β-Mercaptoethanol) diluted with water (1:1, v:v) at a final concentration of 10 mg/ml (w/v). This sample was used to perform the Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and immunoblotting.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The protein profile of pine nuts was analyzed by SDS-PAGE. For this purpose, defatted pine nut flour was suspended in a sample buffer (containing 0.125 M TRIS–HCl pH 6.8, 3.75% glycerol, 1% SDS) diluted with water (1:1, v:v) at a final concentration of 10 mg/ml (w/v) and 10 ml of this sample was loaded in a gel having the following characteristics:

Gradient running gel: 9–19% acrylamide; 0.08-0.17% bis-acrylamide; 0.36 M TRIS–HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N', N'-tetramethylenediamine (TEMED).

Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M TRIS–HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% (TEMED).

Running buffer: 25 mM TRIS, 0.19 M glycine and 0.1% SDS (w/v), pH 8.8.

After the electrophoretic run (90 V at room temperature, for approximately 6 h), gels were dyed with Coomassie Brilliant Blue G-250 by the method of Neuhoff et al. (28). All materials and instruments were purchased from Bio-Rad (Richmond CA, USA).

A prestained molecular-weight marker solution (broad range, Bio-Rad) contained myosin (202.8 kDa), β-galactosidase (115.6 kDa), bovine serum albumin (98.2 kDa), ovalbumin (51.4 kDa), carbonic anhydrase (37.2 kDa), soybean trypsin inhibitor (29 kDa), lysozyme (19.7 kDa), and aprotinin (6.7 kDa).

Immunoblotting

After SDS-PAGE, pine nut proteins were transferred into a Polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) by Western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (150 mM NaCl, 5 mM TRIS, 0.05% Triton-X) to prevent non-specific adsorption of the immunological reagents. Afterward, the membrane was immersed in 10 ml of 0.25% gelatin solution containing 20 μl of human sera. Antigen-IgE complex were detected using 10 μl of goat anti-human IgA polyclonal antibodies labeled with alkaline phosphatase (Chemicon International, Temecula, CA, USA). Finally, after incubation in the bromochloroindolyl phosphate–nitroblue tetrazolium (BCIP/NBT) solution, an intense black-purple precipitate developed at the site of the enzyme binding. The developing solution contained 15% bromochloroindolyl phosphate and 30% nitroblue tetrazolium in alkaline phosphatase buffer (100 mM TRIS, 100 mM sodium chloride, and 5 mM magnesium chloride, pH 9.5).

Results

Two patients reported symptoms of anaphylaxis and three symptoms of urticaria–angioedema. All five children resulted only sensitized to pine nuts and negative to all the other food tested (in two cases there was also a sensitization to *Dermatophagoides pteronyssinus* and grass pollen as shown in Table 1). Figure 1 illustrates the results obtained by SDS-PAGE of pine nuts and the corresponding immunoblotting after incubation of membranes with the sera from the children included in the present study. The electrophoretic pattern of pine nuts under reducing conditions (lane 1) showed 10 protein

Table 1 Clinical and immunological characteristics of the studied patients

Pt.N/age/sex	Symptoms	Pine nut Serum IgE (KU/L)	P-to-P (mm)	Other sensitizations
1/13/F	OAS/V/LE-D	5.3	12	Grass
2/13/M	OAS/U	4.5	5	–
3/13/M	OAS	0.79	10	–
4/13/M	OAS/LE/D	27	5	–
5/5/F	OAS/U	12.1	15	Dust mite

Pt., patient; N, number; P-to-P, Prick-to-Prick; F, female; M, male; OAS, oral allergy syndrome; V, vomit; LE, laryngeal edema; D, Dyspnoea; U, urticaria.

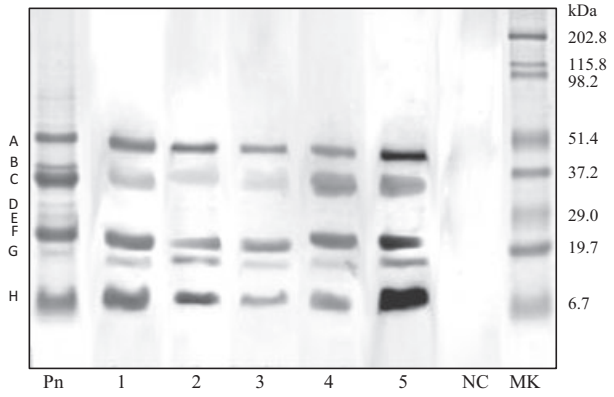

Figure 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of pine seed and immunoblotting obtained after incubation of membranes with five different sera from children monosensitized to pine nut. Lane Pn: Pine nut electrophoretic profile; Lanes 1–5: immunoblotting of pine nut incubated with sera from allergic patients; Lane NC: Negative control; Lane MK: Prestained molecular weight marker; kDa: molecular weight unit.

Table 2 Estimated molecular weight of the major pine nuts proteins

Protein band	Molecular weight (kDa)
Band A	47.1
Band B	38.3
Band C	34.8
Band D	27.7
Band E	24.2
Band F	20.7
Band G	15.4
Band H	6.6

bands with a molecular weight ranging from ~7 to 58 kDa (Table 2). Figure 1 also shows the results obtained by immunoblotting after incubating the membranes, which contain the pine nut proteins (showed in lane 1) with the sera from the five children monosensitized to pine nuts included in this study (lanes 2–6). As expected, all subjects reacted strongly to pine nuts and, furthermore, they presented a similar IgE reactivity pattern to pine nut proteins. Specific IgE reactivity against five pine protein bands were observed in all the children: bands A (~47.1 kDa), C (~34.8 kDa), F (~20.7 kDa), G (~15.4 kDa), and H (~6.6 kDa).

Discussion

Systemic symptoms ranging from urticaria to anaphylactic reactions have been described in patients with pine nut allergy (10–16, 23). Acute anaphylactic reactions after pine nut skin tests have also been reported (29) in the literature. In our study, two children presented anaphylaxis and three urticaria–angioedema after ingestion of pine nuts. All children were sensitized to pine nuts and not to other food tested (in two cases, there was also a sensitization to mixed grass and *D. pteronissuns,* Table 1). Recently, a case report about a young boy with several episodes of anaphylaxis after pine nut ingestion was described (30). Specific IgE to pine nuts and *Artemisia vulgaris* were demonstrated by skin prick tests and *in vitro* CAP, although specific IgE to pine pollen or other nuts were no detected. Immunoblotting of *A. vulgaris* and pine nuts revealed two matching diffuse bands, just below 14 and 30 kDa. The ImmunoCAP inhibition assays showed a complete inhibition of pine nut specific IgE after serum incubation with the *A. vulgaris* extract, indicating cross-reactivity between pine nut and *A. vulgaris*. In our study, however, none of the children were sensitized to *A. vulgaris* even if bands at ~15 and 34 kDa were found. Nevertheless, other further sensitization to inhalants as well as to food allergens in the group of children studied cannot be excluded. Several authors have studied and characterized different allergens in pine nuts through immunoblotting techniques. In this regard, Koepke et al. (14) showed the presence of 30 protein bands by electrophoresis of a pine nut extract in an immunoblot. Three of such protein bands (ranging between 66 and 68 kDa) bound the serum IgE of a patient with pine nut anaphylaxis. Recently, a 7-vicilin-type globulin from the Korean pine was purified and characterized (20).

Allergens from pine nut may induce severe allergic reactions. Ibañez et al. (15) described two cases of young girls with anaphylaxis caused by ingestion of small amounts of pine nuts. The patients had IgE against a specific pine nut protein band with an apparent molecular weight of approximately 17 kDa that could be considered as the main pine nut allergen. As the patients were monosensitized to pine nuts, it was suggested that the 17-kDa protein could be correlated with the most severe clinical symptoms. In this study, five protein bands were recognized by the sera from the children monosensitized to pine nuts. Some of these immunoreactive bands, with a molecular weight ~15. 4 and 47.1 kDa, could correspond to those described in other studies (15, 18, 22, 30). It should be emphasized that the monosensitized children included in this work can be considered a homogeneous and well-characterized group of patients allergic to pine nuts in which all the true allergenic bands could be identified. All five allergic children had specific IgE to the same proteic bands: A (47.1 kDa), C (34.8 kDa), F (20.7 kDa), G (15.4 kDa), H (6.6 kDa). No differences were detected in the IgE response in subjects with anaphylaxis compared with those without anaphylaxis, and for this reason, all the immunoreactive bands may have clinical relevance without apparent correlation with the severity of the symptoms. Further studies will be necessary to investigate the relative clinical importance of the single bands.

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Conflict of interest

The authors declare that they have no conflict of interest.

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